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(54) Title: ERYTHROPOIETIN RECEPTOR

(57) Abstract

DNA sequences and the encoded peptide sequences for murine and human erythropoietin receptor proteins are disclosed. Methods for obtaining and using them, as well as related materials are also disclosed.

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ERYTHROPOIETIN RECEPTOR

Background

Erythropoiesis, the production of red blood cells, occurs in the bone marrow under the physiological control of the hormone erythropoietin (EPO). Erythropoietin is an approximately 34,000 dalton glycoprotein hormone which is synthesized in the kidney, circulates in the plasma, and is excreted in the urine. In response to changes in the level of oxygen in the blood and tissues, erythropoietin appears to stimulate both proliferation and differentiation of immature erythroblasts. It functions as a growth factor, stimulating the mitotic activity of erythroid progenitor cells, such as erythrocyte burst forming and colony-forming units. It also acts as a differentiation factor, triggering transformation of an erythrocyte colony-forming unit into a proerythroblast (Erslev, A., New Eng. J. Med. 316:101-103 (1987)).

Normally, erythropoietin is found in very low concentrations in bodily fluids. However, under conditions of hypoxia, when oxygen transport to erythrocytes is reduced, the concentration of erythropoietin in the blood stream increases. For example, in patients suffering from aplastic anemia, there is an abnormally high concentration of erythropoietin in the urine.

Purified, homogeneous erythropoietin was characterized as a single peak on reverse phase high performance liquid chromatography with a specific activity of at least 160,000 IU per absorbance unit at 280 nanometers. (Hewick, U.S. Patent 4,677,195). A DNA sequence encoding

erythropoietin was purified, cloned and expressed to produce synthetic polypeptides with the same biochemical and immunological properties. (WO 86/03520; Lin, U.S. Patent 4,703,008). A recombinant erythropoietin molecule with oligosaccharides identical to those on the natural material has also been produced. (Sasaki, H., et al., J. Biol. Chem. 262:12059-12076 (1987)).

Despite the availability of purified recombinant erythropoietin, little is known concerning the mechanism of erythropoietin-induced erythroblast proliferation and differentiation. The specific interactions of erythropoietin with progenitors of immature red blood cells, platelets and megakaryocytes remain to be characterized. This is due, at least in part, to the small number of surface erythropoietin receptor molecules on normal erythroblasts and on the erythroleukemia cell line. (Krantz, S.B. and E. Goldwasser, Proc. Natl. Acad. Sci. USA 81:7574-7578 (1984); Branch, D.R. et al., Blood 69:1782-1785 (1987); Mayeux, P. et al., FEBS Letters 211:229-233 (1987); Mufson, R.A. and T.G. Gesner, Blood 69:1485-1490 (1987); Sakaguchi, M. et al., Biochem. Biophys. Res. Commun. 146:7-12 (1987); and Sawyer, S.T. et al., Proc. Natl. Acad. Sci. USA 84:3690-3694 (1987); Sawyer, S.T. et al., J. Biol. Chem. 262:5554-5562 (1987); Todokoro, K. et al., Proc. Natl. Acad. Sci. USA 84:4126-4130 (1988).

Cross-linked complexes between radioiodinated erythropoietin and cell surface proteins suggest that the cell surface proteins comprise two polypeptides having approximate molecular weights of 85,000 daltons and 100,000 daltons, respectively. More recently, the two crosslinked

complexes have been subjected to V8 protease digestion and found to have identical peptide fragments, suggesting that the two EPO-binding polypeptides may be products of the same or very similar genes (Sawyer, et al., 1988). Most cell surface binding studies, however, have revealed a single class of binding sites, averaging 300 to 600 per cell surface, with a Kd of approximately 800 pM (Sawyer, S.R., et al., Proc. Natl. Acad. Sci. USA 84:3690-3694 (1987)). However, EPO-responsive splenic erythroblasts, prepared from mice injected with the anemic strain (FVA) of the Friend leukemia virus, demonstrate a high and a low affinity biding site with dissociation constants of 100 pM and 800 pM, respectively (Sawyer, S.T., et al., J. Biol. Chem. 262:5554-5562 (1987)).

Mouse erythroleukemia cells, although unresponsive to erythropoietin, are a readily available source of EPO binding protein. They have a single class of an EPO binding protein with fewer than 1000 sites per cell and a dissociation constant of 2×10^{-10} M. (Mayeux, P., et al., J. Biol. Chem. 262:13985-13990 (1987); D'Andrea, et al., 1989, submitted). Crosslinking studies with radioiodinated erythropoietin reveal two putative EPO-binding polypeptides with molecular weight of 100,000 and 85,000 daltons.

Knowledge of the mechanism of action of erythropoietin would be of great clinical benefit in treating a number of diseases in which the erythropoietin receptor may be dysfunctional. For instance, it is believed that the erythropoietin receptor is dysfunctional in individuals with Diamond Blackfan anemia, which is a congenital anemia in which the infant is profoundly anemic

and requires red blood cell transfusions and steroid treatments. In polycythemia vera, the erythropoietin receptor may be dysfunctional, but, in this case, it is hyperactive, resulting in a disease characterized in adults by an excess of red blood cell mass. Furthermore, in autoimmune diseases, such as lupus and juvenile rheumatoid arthritis, antibodies to the erythropoietin receptor may account for the anemia associated with these diseases.

Summary of the Invention

This invention encompasses isolated DNAs consisting essentially of nucleotide sequences encoding all or a portion of cell surface receptor proteins (or alleles thereof) for erythropoietin (hereinafter EPO-R), or the functional equivalent thereof. This invention also encompasses recombinant DNA vectors containing the isolated DNAs, as well as the isolated polypeptides encoded by the DNAs (referred to as isolated EPO-R). The invention further encompasses host cells containing the above-described DNAs, methods of producing the encoded EPO-R, methods of treatment which make use of an encoded EPO-R, antibodies specific for an EPO-R or other products which enhance or inhibit EPO-R activity.

The DNA sequence encoding an EPO-R and the encoded polypeptide have several utilities. The DNAs or portions thereof, for instance, can be used to identify the presence, location and/or amount of EPO-R-encoding mRNA or DNA. They can thus be used in further cloning procedures or diagnostic uses, as well as in the production of EPO-R protein by heterologous expression. For example, a DNA

sequence encoding all or a portion of murine EPO-R can be used as a probe for obtaining DNA encoding human EPO-R. The murine DNA will hybridize with human EPO-R RNA transcript, and then this can be reverse transcribed into the DNA sequence encoding all or a portion of human EPO-R. An example of this is described in greater detail below.

EPO-R polypeptides now available, e.g., through heterologous gene expression, can be used as immunogens for the production of antibodies or antibody fragment polypeptides which are specific for EPO-R. Monoclonal antibodies can be producedby conventional methods, e.g., using the standard somatic cell fusion techniques of Kohler and Milstein. A therapeutically effective amount of agonistic antibodies or antibody fragments may be used in pharmaceutical compositions for treating anemic individuals and individuals in whom the EPO-R is dysfunctional. In addition, because of their EPO-binding ability, the polypeptides (EPO-R) can be used to purify EPO. For example, they can be used directly in batch form or can be immobilized in a column. Similarly, immobilized EPO can be used to purify EPO-R proteins or EPO-binding portions thereof as well as cells expressing membrane-bound EPO-R proteins. These and other aspects of the invention are further discussed below.

Furthermore, the understanding and characterization of the erythropoietin receptor may result in the elucidation of the mechanism by which erythropoietin stimulates erythropoiesis. This may advance the study of several human diseases which are believed to be the result of a defective EPO-R.

Brief Description of the Drawings

Figure 1 is the murine EPO receptor cDNA (clone 190) nucleotide and predicted amino acid sequences. The hydrophobic putative signal peptide ends at residue 24 (arrow) and the single transmembrane region is underlined. The sites of potential asparagine-linked glycosylation are boxed.

Figure 2 is the corresponding cDNA and peptide sequence for human EPO-R.

For a hydropathy plot of the predicted of amino acid sequence murine EPO-receptor; a saturation curve showing the binding of iodinated EPO to MEL cells; a saturation curve showing the binding of iodinated EPO to COS cells expressing recombinant murine EPO-receptors; a graph showing the inhibition of EPO binding to MEL monolayers or to COS-EPO-R transfectants by monoclonal antibodies against recombinant human EPO; a photograph of an RNA blot analysis of EPO-R receptor mRNA; a photograph showing crosslinking of radiolabeled erythropoietin to EPO-receptor expressed in COS cells; see D'Andrea, et al., Cell 57:277-285 (April 1989), which is incorporated herein by reference.

Detailed Description of the Invention

This invention relates to DNA sequences encoding an EPO-R of mammalian origin, to the encoded EPO-R proteins and to their use in detecting EPO-R dysfunction, as well as in treating individuals in whom such dysfunction occurs. Other aspects of the invention are also discussed infra.

The following is a description of cloning and expression of DNAs encoding EPO-R, of the characterization/assessment of EPO-R and of uses of EPO-R proteins or related materials for diagnostic, therapeutic and other purposes.

This invention encompasses DNA which comprises the nucleotide sequences depicted in Figures 1 or 2 or which otherwise encodes a peptide sequence containing either of the mature peptide sequences depicted therein. This invention further encompasses DNA which is capable (or would be so, but for the incorporation of synonymous codons) of hybridizing to the aforesaid DNA, or to the corresponding cloned human genomic or cDNA (e.g., as shown in the Figures or embodied by the DNAs deposited with the ATCC and discussed infra), especially under stringent conditions, and which codes, on expression, preferably in mammalian host cells, EPO receptor protein, or a portion thereof, e.g., which binds to EPO, as can be readily determined, e.g., by methods disclosed in detail hereinafter. Thus, this invention specifically encompasses DNA which comprises the nucleotide sequence of cloned human EPO-R DNA and/or which codes on expression a human EPO receptor protein or a portion thereof. The above-mentioned DNAs may be cDNAs or other intronless DNAs, or may be isolated genomic DNAs. The DNAs may alternatively be synthesized DNAs which encode a peptide sequence also encoded by one or more of the aforesaid DNAs.

In many embodiments, such EPO receptor-encoding DNA is covalently linked to heterologous DNA, i.e., DNA from sources other than that of the EPO receptor-encoding DNA. Typically, such constructs comprise plasmids or vectors containing

the DNA of this invention linked with vector DNA and various genetic elements advantageous for selection, transcription control, amplification, etc. as described in greater detail below.

A detailed description of methods for obtaining DNA of this invention are provided below. However, it should be noted that given the nucleotide sequence information provided in the accompanying Figures, one may now simply use a synthetic oligonucleotide incorporating part or all of the sequence of Figure 1 or 2 as a hybridization probe in order to more readily identify and isolate a mammalian, preferably human, genomic DNA or cDNA encoding the EPO receptor. Alternatively, one may simply use the cloned human DNA as a hybridization probe, as described in further detail below. It should also be noted that DNAs encompassing the sequences depicted in the Figures may be synthesized as a set of overlapping oligonucleotides which may then be annealed and ligated, all by purely conventional methods, in order to produce a corresponding synthetic DNA. We also note that oligonucleotides designed based on information disclosed herein may be employed with now conventional polymerase chain reaction materials and methods to identify other clones, and further, that, as indicated below, certain EPO-R clones are on deposit at the American Type Culture Collection, Jefferson Davis Highway, Rockville, MD USA.

Murine clone 190 inserted within the cloning site of mammalian expression vector pXM (described in Yang, et al., 1986) has been deposited with the American Type Culture Collection as pMuEpo-R190 under ATCC No. 40546. Two cloned human genomic EPO

receptor DNA fragments inserted within the cloning site of a lambda phage vector have also been deposited as HuEPOR 3-2 and HuEPOR 2-1A under ATCC No. 40547 and ATCC No. 40548, respectively.

DNAs of this invention are preferably expressed in mammalian host cells, although bacterial, yeast and insect cell expression may be readily effected using purely conventional methods and materials.

Eukaryotic, preferably mammalian, expression vectors into which the DNAs of this invention may be inserted (with or without synthetic linkers, as desired or necessary) may be synthesized by techniques well known to those skilled in this art. The components of the vectors such as the bacterial replicons, selection genes, enhancers, promoters, and the like may be obtained from natural sources or synthesized by known procedures. See, e.g., Kaufman et al., J. Mol. Biol., 159:601-621 (1982); Kaufman, Proc. Natl. Acad. Sci., 82:689-693 (1985). Eucaryotic expression vectors useful in producing variants of this invention may also contain inducible promoters or comprise inducible expression systems as are known in the art. See e.g., "High Level Inducible Expression of Heterologous Genes," International Application No. PCT/US87/01871, the contents of which are incorporated herein by reference.

Established cell lines, including transformed cell lines, are suitable as hosts. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants (including relatively undifferentiated cells such as haematopoetic stem cells) are also suitable. Candidate cells need not be genotypically deficient

in the selection gene so long as the selection gene is dominantly acting.

The host cells preferably will be established mammalian cell lines. For stable integration of the vector DNA into chromosomal DNA, and for subsequent amplification of the integrated vector DNA, both by conventional methods, CHO (Chinese Hamster Ovary) cells are currently considered preferred. Alternatively, the vector DNA may include all or part of the bovine papilloma virus genome (Lusky et al., Cell, 36:391-401 (1984) and be carried in cell lines such as C127 mouse cells as a stable episomal element. Other usable mammalian cell lines include HeLa, COS-1 monkey cells, melanoma cell lines, such as Bowes cells, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines and the like.

Plasmid pMT2 may be obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods and then ligated to DNAs of this invention. pXM or pMT21, derivatives of pMT2, may also be used as alternatives to pMT2. Of course, one seeking to express a DNA of this invention would most likely prefer to use an expression vector from his or her own laboratory.

Transformants, preferably of mammalian cells such as COS or CHO cells, containing and capable of expressing a DNA of this invention provide cell lines useful for the production of EPO receptor proteins. CHO cells, in particular, allow the production, identification and recovery of stably transformed cell lines containing amplified gene copy number of the EPO receptor DNA and expressing recombinant EPO receptor protein in yields permitting recovery thereof from the cell cultures. Suitable transformation, selection, amplification and cell culture methods are conventional in this art.

It should be noted that the presence of EPO receptor protein may be conveniently detected by use of radiolabeled or otherwise labeled EPO protein. EPO protein of course may be obtained by methods now well known in the art. See e.g., Published International Application No. WO 86/03520. In addition, EPO protein is now commercially available.

EPO receptor proteins may be recovered from the cell cultures by conventional means, including affinity chromatography using immobilized EPO protein as the affinity reagent, reverse phase HPLC, and other techniques conventional for recovery of membrane bound protein. Cells containing the proteins can be used to recover the EPO receptor protein.

Thus, this invention provides for the first time EPO receptor protein, preferably human, free or substantially free from other proteins with which it is otherwise associated in nature.

Preferably the EPO receptor protein is at least about 90%, and more preferably more than 95% free on wt/wt basis from such other proteins.

EPO receptor proteins of this invention may be used for the production for the first time of idiotypic or antiidiotypic antibodies to the receptor, whether polyclonal or monoclonal, which antibodies (as well as portions thereof which also bind to the antigen) are also encompassed by this invention - whether produced by hybridomas or by heterologous expression of cloned DNA. Such antibodies may be useful therapeutically in the treatment of anemias. Antibodies may also be raised against the EPO-R protein or against an EPO-R protein complexed with EPO. Some antibodies raised against receptor or receptor-ligand complex may be agonistic or antagonistic. Both would have potential therapeutic uses: agonists for anemic states, and antagonists for conditions such as polycythemia vera. The antigen may comprise a purified receptor protein, purified receptor protein reconstituted in lipid vesicles, preparations of membrane containing the receptor- with or without complexed ligand. The receptor protein may be intact, portions of it may be used, or soluble forms of receptor (lacking part or all of the transmembrane region) may be employed.

The EPO receptor proteins or portions thereof may also be used as an affinity reagent for the identification (e.g., in EPO assays) or purification of EPO. Each of these uses is based on the binding affinity of EPO for its receptor. In the case of purification of EPO, it should be noted that the murine receptor protein may be as efficient as or better than the corresponding

human receptor protein. In the case of assays, it should be noted that the EPO receptor protein may be radiolabeled or may be expressed as a fusion protein with beta-galactosidase, alkaline phosphatase or other enzymatic labels for convenient monitoring. Additionally, it should be noted that truncated forms of the EPO receptor proteins which retain the EPO binding site may also be used for such purposes.

For instance, it should be noted that DNAs of this invention may be modified by deletion of part or preferably all of the region encoding the membrane-spanning protein domain, or may be modified by deletion of part or all of the region encoding the protein domains preceding (N-terminal to) the extracellular domains of the receptor. Expression of such modified DNAs by the methods disclosed herein should permit the production of EPO receptor protein variants which retain EPO binding activity but are no longer membrane bound. Such variants may be secreted from the producing cells and recovered from the culture media.

Additionally, it should be noted that EPO-R proteins of this invention may also be used in the screening of other agents, including modified forms of EPO, which retain EPO receptor binding activity. Binding assays using a desired binding protein, immobilized or not, are well known in the art and may be used for this purpose using an EPO receptor protein of this invention. Compounds exhibiting detectable binding to EPO-R proteins may thus be identified and then secondarily screened in the now well known EPO activity assays, preferably <u>in vivo</u> activity assays. By these means compounds having

EPO activity which may be suitable as alternatives to EPO may be identified.

The development of EPO-dependent cell lines, another aspect of this invention, provides another approach to such screening. EPO-dependent cell lines may be prepared by transfecting or transforming a cell line dependent on some other growth or proliferation factor, such as IL-3, with a DNA sequence of this invention such that the cell expresses the encoded EPO-R protein. Essentially, the strategy is to have an EPO-R protein provide an alternative transducer of signals for cell proliferation and/or differentiation. For example, a murine IL-3-dependent cell line was transformed with pMuEpo-R190 which contains the murine EPO-R cDNA of Figure 1 operably linked to transcriptional and translational regulatory signals such that the encoded EPO-R protein was expressed. The resultant cells were found to bind to and incorporate radiolabeled EPO. Their continued growth was found to be dependent now on the presence of EPO (or IL-3 - rather than exclusively IL-3) in the culture medium. These cells were shown to express the heterologous EPO-R gene by Northern blot and Western blot analysis. This demonstrated that heterologous expression of an EPO-R encoding DNA led to the expression of functionally active receptor protein - i.e., to receptor protein which binds EPO and transduces a signal into the cell. EPO-dependent cell lines may also be prepared from cells dependent on factors other than IL-3, from human or other non-murine mammalian cells, and using human or other EPO-R-encoding DNAs of the invention which encode proteins retaining the EPO-binding, transmembrane and intracellular domains.

Amplification of gene copy number should that be desired, may be effected by conventional methods using a dominant acting marker gene, e.g., ADA. Selection may be readily accomplished based on cell survival in the presence of EPO and cell death in the absence of appropriate growth factor.

Such cells provide a more convenient screening reagent for EPO agonists or antagonists. According to this method, EPO-dependent cells are grown in culture medium containing a test material, e.g. a polypeptide, antibody, antibody fragment or other organic molecule. These cells have a non-detectable rate of transformation to factor independence - that is, 100% of the cells die in about 12 hours in the absence of EPO. Only cells cultured in the presence of EPO or an EPO agonist survive. Thus, the cells are cultured in the presence of the test material for a period of time at least as long as required for substantially all of the cells to die in the absence of EPO or an EPO agonist, e.g. at least about 12 hours, although the precise amount of time should vary with different EPO-dependent cell lines. Culture of the cells in the presence and absence of EPO can serve as controls. Cells which continue to grow in the presence of a test material and absence of added EPO in the culture medium are identified and the test material is thus identified as an EPO agonist. Of course, a test material which supports the growth of an EPO-dependent cell line in the absence of added EPO may have its identity as an EPO agonist secondarily confirmed by EPO activity assay, as previously mentioned. This invention encompasses materials, preferably antibodies, fragments or portions of antibodies,

non-proteinaceous materials, non-glycosylated proteinaceous materials, peptides (preferably about 20 amino acids or shorter in length), and proteins having substantially no homology to human EPO (preferably less than 75% homology, more preferably less than 50% homology, and most preferably less than 20% homology to human EPO peptide sequence) which are capable of supporting the growth of an EPO-dependant cell line in the absence of added EPO. This invention also encompasses such materials first identified as EPO agonists by a method of this invention, e.g., using an EPO-R protein in a binding assay, in the context of an EPO-dependent cell line or otherwise.

Construction of a cDNA Library

A cDNA library from uninduced MEL cells which express the EPO-R, was expressed and analyzed as described in the following sections. From uninduced MEL cells, which express approximately 7000 receptors per cell surface, a cDNA library was prepared in the mammalian expression vector pXM (Yang, Y. et al., Cell, 47:3-10 (1986)). The library contained approximately 800,000 independent clones. These were plated into pools, each with approximately 1000 different recombinant bacterial colonies. Plasmid minipreps were prepared from each pool (Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)) and plasmid DNA was transfected in duplicate into COS monolayers by the DEAE dextran method (Sompayrac, L.M. and K.J. Danna, Proc. Natl. Acad. Sci., USA, 78:7575-7578 (1981)).

After 72 hours, at the time of peak heterologous protein synthesis, the uptake of radioiodinated recombinant EPO was measured at 37°C for 90 minutes. The EPO was internalized by receptor-mediated endocytosis, and a greater signal was achieved than by measuring binding at 4°C to surface EPO-R's. After uptake, the COS monolayers were washed extensively, and counted in a gamma counter. Under these conditions, background binding of radiolabeled EPO to a 10 centimeter plate of confluent COS cell transfectants was approximately 800 counts per minute. The two positive pools of recombinant plasmids (out of 200 pools, or a total of 200,000 recombinant clones tested), when transfected into COS monolayers, yielded binding of 1200 to 3000 cpm. These two pools of plasmids, numbers 141 and 190, were partitioned into subpools until a single cDNA clone capable of causing COS cells to bind and uptake ^{125}I-EPO was obtained from each pool. When two micrograms of either of the purified single clones was transfected into COS monolayers, radiolabeled EPO uptake at 37°C was greater than 200,000 cpm.

Cloning of the EPO Receptor cDNA

Two cDNA clones encoding EPO-R have been isolated from a pXM expression library made from uninduced murine erythroleukemia (MEL) cells and identified by screening COS cells transfectants for binding and uptake of radioiodinated recombinant human erythropoietin. As inferred from the cDNA sequence, the murine EPO-R is a 507 amino acid polypeptide with a single membrane spanning domain. It shows no similarities to known proteins or nucleic acid sequences. Although the MEL cell

EPO-R has a single affinity (approximately 240 pM), the EPO-R cDNA, expressed in COS cells, generates both a high affinity receptor (30 pM) and a low affinity receptor (210 pM). The isolation of the two independent cDNA clones from a MEL cell library, when transfected alone into COS cells, encode a functional EPO receptor.

cDNA Characterization and Sequence

The inserts of the two specific EPO receptor cDNA clones were excised by Kpn digestion and analyzed by agarose gel electrophoresis. Clone 141 had a slightly longer insert (1.9 kb) than clone 190 (1.8 kb). The restriction maps of both clones were identical; clone 141 was 100 bp longer at the 5' terminus (D'Andrea, et al., Cell (1989) supra). Pst-digested cDNA fragments from the two clones were subcloned into M13mp18 or M13mp19 vectors and the nucleotide sequence was determined by the chain termination method (Sanger, F. et al., Proc. Natl. Acad. Sci., USA, 74:5463-5467 (1977)). Both strands of the cDNA clones sequenced were shown to be co-linear except for a two-base pair deletion at nucleotide position 1333-1335 (Figure 1, arrow). To further evaluate this discrepancy between the two cDNA clones, the normal mouse structural gene for the EPO-R was cloned and sequenced. The coding region for the normal mouse gene agrees with the cDNA sequence shown for clone 190.

The 1773 base cDNA nucleotide sequence of clone 190, shown in Figure 1, reveals a single open reading frame of 507 amino acids. The 3' untranslated region extends for an additional 167 bases and ends with a poly(A) tail. The first initiator codon (ATG) in clone 190 is 43 bases from

the 5' end. No consensus sequence typical of translation initiation (Kozak, M., Nucl. Acids Res., 15:8125-8148 (1987)) was found in the 5' untranslated region.

Since the clones were isolated by an expression strategy, the clones are complete, and encode a polypeptide capable of normal processing, cell surface localization, and binding of EPO. Consistent with the assignment of the first ATG as initiator (Figure 1), there are three stop codons in frame 5' to the first methionine of the predicted amino acid sequence. The N-terminal 24 residues have all of the features of a typical signal sequence. The hydrophobicity plot (Kyte, J. and R.F. Doolittle, J. Mol. Biol., 157:105-132 (1982)) predicts a single 23 amino acid membrane-spanning alph-helical segment from amino acids 248-271 (D'Andrea, et al., Cell (1989) supra). The putative transmembrane region is followed by a sequence of mostly basic residues. This feature is common to the cytosolic face of the membrane-spanning segments of many proteins. This suggests an amino-terminus-exoplasmic-carboxy-terminus-cytoplasmic orientation, or a so-called type I transmembrane protein.

There are two potential sites of N-linked glycosylation, one in the putative extracellular domain and one in the putative cytoplasmic domain. Also, 12% of the amino acids are serine residues and 5% are threonine, making extensive O-linked glycosylation a possibility (Russell, D.W. et al., Cell, 37:577-585 (1984)). The discrepancy between the sizes of the putative receptor polypeptides, observed by crosslinking studies - 85,000 or 100,000 daltons - and the predicted 57,000 mw

predicted from the cDNA sequence could be accounted for by glycosylation. Also, there is a high frequency of proline residues (10% of total amino acids), present throughout the sequence, suggesting an absence of alpha-helical secondary structure.

The nucleotide and peptide sequences revealed no signific-ant homology to any other cloned genes in the Gen bank data base. The deduced amino acid sequence of the EPO-R reveals several overall features common to other growth factor receptors. The extracellular domain (amino acid 25-248) should contain the EPO binding region. Despite the existence of five cysteine residues within this region, there is no evidence of the conserved disul-fide loops characteristic of receptors of the immunoglobulin superfamily (Sims, J.E. et al., Science, 241:585-589 (1988)). The intracellular domain (amino acids 272-507) could serve a role in signal transduction. Despite its large size, the cytoplasmic domain has no apparent sequence homology with the catalytic domain of any growth factor receptor known to be a tyrosine kinase (Hanks, S.K. et al., Science, 241:42-52 (1988)).

Binding Characteristics of the Recombinant EPO Receptor

The binding affinity of the recombinant EPO receptor, expressed in transfected COS cells, is similar to that of the receptor on MEL cells (see D'Andrea, et al., Cell (1989) supra). For these experiments, MEL cells, grown as monolayers on fibronectin coated dishes, were incubated for eight hours at 4°C with ^{125}I EPO. Specific binding and nonspecific binding of [^{125}I]-EPO, measured in the presence of 100 nM unlabeled EPO was determined.

Non-specific binding was approximately half of the total (= specific plus non-specific) binding. Because of the relatively low number of EPO-R on MEL cells and, therefore, the small specific binding observed at low concentrations of [^{125}I EPO], in the 10-50 pM range, it is not possible to rule out a high affinity EPO-R in this range.

Binding was compared for COS transfectants expressing EPO-R (specific binding) versus COS cells mock-transfected with the pXM plasmic without the EPO-R cDNA insert (non-specific binding). At all EPO concentrations, specific binding was approximately 40 times the binding to mock-transfected cells. By the above criterion, over 85% of this binding is deemed specific, though the "non-specific" binding could represent low-affinity binding to the transfected EPO receptor. Scatchard analysis of the specific binding revealed the presence of two receptor species having apparent dissociation constants of two affinities, 30 pM and 210 pM, respectively. Based on immunofluorescence of COS monolayers transfected in parallel with H1 cDNA, 10% of transfected COS cells expressed recombinant surface proteins. Therefore, each COS EPO-R transfectant expressed approximately 210,000 EPO-R cell surface molecules, 16% as the high affinity class and 84% as the lower affinity class.

Attachment of radioiodinated EPO to MEL cells or other cells bearing EPO-receptors is 5-10 fold greater at 37°C versus 4°C, suggesting that endocytosis of EPO occurs (Sawyer, S.T. et al., Proc. Natl. Acad. Sci., USA, 84:3690-3694 (1987); Mufson R.A. and T.G. Gesner, Blood, 69:1485-1490 (1987)). During cloning, the transfected cells

were incubated at 37°C while selecting clones capable of undergoing endocytosis. In COS cells expressing transfected clone 190 cDNA, cell attachment of radioiodinated EPO after 90 minutes at 37°C was 10 times the binding to the cell surface which occurred during 8 hours at 4°C, suggesting that the recombinant EPO receptor, expressed in COS cells, will undergo endocytosis.

Antibody Inhibition of EPO Binding

Four high affinity monoclonal antibodies against recombinant human EPO were used to determine the specificity of binding be-tween radiolabeled EPO and the recombinant EPO receptor expressed in COS cells. All four monoclonal antibodies bind to EPO with Kd values from about 0.5 nM to 50 nM. To measure the effects of these antibodies on EPO-receptor interaction, radioiodinated EPO was first incubated with enough antibody to immunoadsorb 100% of the EPO. The MEL cells were grown as monolayers on fibronectin-coated petri dishes and incubated in 4°C with radiolabeled EPO in the presence (non-specific binding) or absence (total binding) of unlabeled EPO. Two monoclonal antibodies (MoAb #1 and MoAb #4) did not block binding of EPO to its MEL receptor, but two others (MoAb #2 and MoAb #3) did inhibit in a dose dependent manner. This same antibody inhibition pattern was replicated by the COS transfectants (D'Andrea, et al., Cell (1989) supra) suggesting that EPO binds to the recombinant EPO receptor with the same orientation as it binds to the EPO receptor on MEL cells.

Tissue Specific Expression of EPO-Receptor

As examined by Northern blot analysis (D'Andrea, et al., Cell (1989) supra), erythropoietin receptor transcripts were only identified in cells of the erythroid lineage. Poly A selected RNA from either MEL cells, in normal splenic erythroblasts recovered from a mouse treated with phenylhydrazine, or HCD57 cells, an MEL cell line which shows absolute dependence on EPO for viability. All cells contained EPO-R transcripts. The full length transcript of 2.1 kb is slightly larger than the 1.8 kb and 1.9 kb cDNA class isolated by expression cloning. Poly A selected RNA from normal mouse tissues including brain, liver, and kidney (D'Andrea, et al., Cell (1989) supra, lanes 4-6 respectively) show no hybridization with the full length ^{32}P-labeled cDNA probe. Also, Southern blot analysis of both mouse and human genomic DNA suggests that the EPO-R transcript is most likely the product of a single gene.

The EPO receptor from MEL cells was cloned by expression. The cloning was improved by two novel features not formerly employed in other COS cell cloning strategies. First, the COS transfectants were assayed for uptake of radioiodinated EPO at 37°C, as opposed to surface binding at 4°C. This improved the signal and allowed screening of larger pools of recombinant plasmids (approximately 1000) per transfection. Secondly, the low level of non-specific binding of radioiodinated EPO to COS transfectants and the small standard deviation from one negative pool to the next (800 + 134 cpm) allowed the identification of two positive plasmic pools with the use of a gamma counter alone. In

contrast, for radioiodinated ligands with higher non-specific binding, identification of a positive pool requires autoradio-graphy (Sims, J.E. et al., Science, 241:585-589 (1989)).

Although the EPO-receptor cDNA cloned encodes a single polypeptide, the COS transfectants demonstrate a high and a low affinity receptor. The generation of two affinity states by a single receptor polypeptide can be explained in multiple ways. First, the EPO-R polypeptide may undergo differential carbohydrate processing, generating two different products with different affinities. Second, the EPO-receptor may undergo phosphorylation of the cytoplasmic domain, generating two receptor affinities. Phosphorylation of the EGF-receptor, for example, is known to decrease the binding affinity for EGF. Third, the EPO-receptor polypeptide may interact with some endogenous COS cell polypeptide which is absent in MEL cells, thus generating two distinct affinities. The interaction of two discrete polypeptides to generate a high affinity receptor in some cell lines has been described for the IL-2 receptor. Fourth, the EPO polypeptide may undergo dimerization, generating a distinct affinity for both the monomeric and dimeric forms. This is appealing because the cross-linked complex of an EPO-R polypeptide dimer EPO would sum to approximately 140 kb, the size of the crosslinked complex for both normal erythroid cells (Sawyer, S.T. et al., Proc. Natl. Acad. Sci., USA, 84:3690-3694 (1987) and for EPO-R COS transfectants.

Several lines of evidence suggest that the high affinity EPO receptor is the physiologically important receptor. First, mouse splenic

erythroblasts which are responsive to EPO have both high and low affinity receptors, while EPO unresponsive MEL cells have only the low affinity receptor. Second, the dissociation constant of the high affinity receptor (30 pM) correlates well with the typical concentration of erythropoietin in mouse and human serum, suggesting that occupancy of the high affinity receptor is all that is required for signal transduction. Third, treatment of purified mouse CFU-E's with EPO results in a selective decrease of the high affinity receptor only.

Importantly, MEL cells, although expressing EPO-R's of comparable number and affinity to normal erythroblasts, do not respond to EPO by either proliferation or differentiation. MEL cells are derived from mice infected with Friend virus complex which is comprised of both the Friend leukemia virus and the replication incompetent spleen focus forming unit (SFFUp). The transformation by Friend virus complex may bypass the EPO receptor signal transduction pathway. The envelope protein encoded by the SFFUp may interact with the EPO receptor, generating constitutive signal transduction in the absence of EPO. Alternatively, but less likely, the MEL EPO-R may have undergone a mutation during MEL cell generation rendering it able to transduce a growth-promoting signal even in the absence of EPO.

Cells and Cell Culture

Mouse erythroleukemia (MEL cells, subclone 745, were obtained. The cells were cultured in suspension in Dulbecco's modified Eagle's medium (DMEM) plus 13% heat-inactivated (HI)FCS in a humid

CO_2 (5% CO_2 and 95% air) incubator at 37°C. For monolayer growth, MEL cells were attached to 60 nm petri dishes precoated with fibronectin (Patel and Lodish, 1987). COS-1 cells were routinely maintained in DMEM plus 10% HIFCS in a human CO_2 and 90% air) incubator at 37°C.

Construction of pXM cDNA Library

One milligram of total RNA, prepared from uninduced MEL cells grown in suspension, was isolated by the guanidinium isothiocyante procedure (Chirgwin et al., Biochemistry, 18:5294-5299 (1979)). Five micrograms poly A selected mRNA was converted to double-stranded cDNA as described previously (Wong et al., Science, 228:810-815 (1985)). Blunt end cDNA was ligated to semi-Xho adapters, non-ligated adapters were removed by CL-4B sepharose chromatography, and semi-Xho adapted cDNA was ligated into the COS-1 cell expression vector pXM, prepared as described (Yang et al., Cell, 47:3-10 (1986)). The ligation mixture of approximately 800,000 ampicillin-resistant colonies.

DNA Preparation

Approximately 200,000 bacterial colonies from the library were replicated onto nitrocellulose filters, plated at a density of approximately 1000 colonies per plate. Nitrocellulose replicas of each pool of 1000 colonies were made, incubated on LB plates with 5% glycerol for 30 minutes at 37°C, and stored at -80°C. The master filter from each pool was grown over 24 hours and bacterial colonies were scraped into L broth. Plasmid DNAs were

isolated by a modification of the alkaline lysis technique (Maniatis, T. et al., Molecular Cloning: A Laboratory Manual (1982).

COS-1 Cell Transfection

Two to five micrograms of each plasmid pool was used to transfect each of two COS-1 monolayers grown on 10 cm tissue culture dishes. Transfection was by a DEAE-dextran protocol modified by a 0.1 mM chloroquine treatment (Sompayrac and Danna, PNAS, 78:7575-7578 (1981); Luthman and Magnusson, Nucl. Acids Res., 11:1295-1308 (1983)). After 72 hours, media was removed from the transfected COS-1 cells, and transfected monolayers were assayed for radioiodinated EPO binding.

Radioiodination of Recombinant Human Erythropoietin

Highly purified recombinant human erythropoietin was stored at -80°C in a 5.8 mM PO_4, 0.4 M NaCl, pH 7.3 buffer at 0.985 mg/ml, as determined by amino acid analysis. EPO was radioiodinated by Bilheimer's modification (Bilheimer et al., 1972) of the iodine monochloride technique of MacFarlane (MacFarlane, 1958) in the presence of $Ma^{125}I$ (Amersham). Specific activities ranged from 500 to 1000 cpm per fmole.

Screening of COS-1 Transfectants

Duplicate monolayers of COS-1 transfectants, ground on 10 cm tissue culture dishes were assayed by uptake of radioiodinated EPO. Monolayers were washed three times with Hanks Balanced Salts Solution containing 25 mM Hepes, pH = 7.5 (HBS/Hepes) at 23°C. Radioiodinated EPO was added to each dish in ligand binding buffer (LBB) which

was 1 x DME, 1% bovine serum albumin, 25 mM Hepes, pH = 7.5 and 2 x 10^6 cpm ^{125}I-EPO in 3 milliliters LBB. Monolayers were incubated for 90 minutes at 37°C with gentle rocking. Unbound radioiodinated EPO was removed and monolayers were washed three times with HBS/Hepes at 23C. Each monolayer was the solubilized in three milliliters of 1 N NaOH and counted using an AUTO-GAMMA 500 c/800 c gamma counter (Packard). For monolayers transfected with negative pools of plasmids, background binding of radioiodinated erythropoietin was 800 ± 134 cpm. Two positive pools were identified which yielded COS-1 monolayer binding of approximately 1200 cpm. After identification of a positive pool, a frozen replica on nitrocellulose, containing approximately 1000 colonies, was thawed and cut into approximately 30 sections. Minipreps were prepared from individual sections and transfected into COS cells. Individual colonies were next picked from the positive sections. After a final round of miniprep and COS transfection, the single clone was recovered.

Erythropoietin Surface Binding Assay

Confluent monolayers of MEL cells, grown in 60 nm bacteriophage petri dishes pre-coated with fibronectin, were prepared (Patel and Lodish, J. Cell. Biol., 105:3105-3118 (1987)). Confluent monolayers of COS-1 cells, transfected by the DEAE dextran method 72 hours before the binding assay with either two micrograms of pSM without cDNA insert (mock-transfected) per monolayer, were prepared. Monolayers were washed at 4°C with HBS/Hepes three times. Radioiodinated EPO (10 pM to 2nM range) was added to each monolayer in LBB.

Incubations were performed at 4°C for eight hours with gentle rocking. Monolayers were washed, solubilized, and counted in the gamma counter as described. Assays for nonspecific binding were performed in which the assay mixture contained a 100-fold excess (100 nM) of unlabeled erythropoietin. The radioactivity bound in the assays with excess unlabeled erythropoietin (nonspecific binding) was subtracted from the total binding to yield the specific binding. All binding assays were run in duplicate.

Calculation of transfection efficiency

Two micrograms of a plasmic clone, containing the cDNA encoding the H1 subunit of the human asialoglycoprotein receptor in the vector, pXM was transfected into COS-1 monolayers. After 72 hours, at the time of peak heterologous protein expression, COS transfectants were labeled sequentially with an anti-peptide antibody against the carboxy terminus of H1 (Bischoff and Lodish, J. Biol. Chem., 262:11825-11832 (1987)) followed by a fluorescein conjugated goat anti-rabbit antibody. The percent of transfected cells was determined using a fluorescent microscope.

Hybridization techniques

Southern and Northern blot hybridizations were performed according to standard techniques described elsewhere (Maniatis et al., Molecular Cloning: A Laboratory Manual (1982)). A cDNA probe was prepared from the full length Kpn fragment of clone 190 by the random oligonucleotide primer labeling method (Feinberg and Vogelstein, Anal. Biochem., 132:6-13 (1983)).

Isolation of the Human Gene for an Erythropoietin Receptor

pMuEPO-R190 (ATCC No. 40546 is plasmid pXM containing a 1.8 kb murine cDNA fragment encoding an erythropoietin receptor. This cDNA fragment can be excised from plasmid pMuEPO-R190 by the restriction enzyme KpnI. Using this DNA fragment as a probe, it is possible to isolate the human gene for an EPO-R protein.

There is similarity in the nucleic acid sequence and gene structure, e.g., intron-exon arrangement, between human and murine EPO receptor genes. The degree of similarity between human and mouse genes determines the hybridization conditions under which a DNA fragment encoding the murine EPO-R will hybridize to the human EPO-R gene. These hybridization conditions can readily be empirically determined by conventional means well known in the art.

Human and mouse genomic DNA are analyzed in parallel by Southern blot hybridization. The human and mouse genomic DNA are treated with various restriction enzymes, e.g., BamHI, EcoRI, HindIII, and chromatographed on an agarose gel by electrophor-esis. The restriction enzyme digested DNA is separated by size on the agarose gel. The DNA fragments are transferred by standard techniques to a nitrocellulose filter, and baked in vacuo at 80°C for 1.5 hours to secure the DNA to the filter. The filter was then incubated in a standard DNA hybridization mixture of 6XSSC, 5X Denhardts', 100 ug/ml denatured salmon sperm DNA, 50 mM Tris pH 7.5, with denatured ^{32}P radiolabeled murine erythropoietin receptor DNA fragment. The murine erythropoietin receptor fragment can be

radiolabeled by numerous techniques described in Maniatis, et al., Molecular Cloning: A Laboratory Manual (1982) including the technique of Feinberg and Vogelstein, Anal. Biochem., 132:6-13 (1983). The murine erythropoietin receptor fragment was radiolabeled to a specific activity of about 10 dpm/ug of DNA. The radiolabeled murine erythropoietin receptor probe was added at a concentration of 10^6 dpm per ml of hybridization mix. The hybridization temperature was determined by the degree of similarity in nucleic acid sequence between murine and human erythropoietin receptor genes. If the similarity between mouse and human erythropoietin receptor genes is high, on the order of 90%, then stringent hybridization conditions can be used.

A hybridization temperature of 48°C was used in the example below. After a hybridization period of 18 hours, the unhybridized probe and nonspecific hybridization was removed by washing the filter in various concentrations of SSC at various temperatures. Wash conditions of 0.5XSSC for 0.5 hours at 55°C were found to remove background nonspecific hybridization by the radiolabeled probe. The nitrocellulose filter (southern blot) was autoradiographed on film. Discrete bands were detected in both mouse and human genomic DNA. Analysis of the mouse genomic DNA that had been subjected to different restriction enzyme treatment revealed there was a single gene for the erythropoietin receptor per haploid genome. In the human genomic DNA, fainter bands above a nonspecific background were detected. Therefore, the human gene for an erythropoietin receptor was

identified by using the murine erythropoietin receptor cDNA as a probe.

A commercially available human genomic library in phage Lambda Fix was obtained from Stratagene. The Lambda Fix human genomic library is screened for the human erythropoietin receptor gene by infecting E. coli strain LE392 with 6 x 10^5 pfu and plating the infected cells on 15 cm NZCYM agar plates at a density of 1.5 X 10^4 pfu per plate. These phages are screened in duplicate using the procedure of Benton and Davis (Molecular Cloning: A Laboratory Manual (1982)) with the 1.8 kb murine erythropoietin receptor cDNA fragment excised by KpnI digestion from pMuEPO-R190. The 1.8 kb murine EPO-R cDNA fragment was radiolabeled as described above. The human genomic library was screened using the standard hybridization mixture (above) at 48°C for 18 hours. The nonspecific hybridization signals are removed by washing the filters at 55°C in 0.5XSSC for 1 hour.

Phages exhibiting a strong hybridization signal are picked and replated at about 100 pfu per 10 cm NZCY, plate, and screened in duplicate again by the Benton and Davis procedure using the murine erythropoietin receptor cDNA fragments as a probe.

Two independent phages huEPOR-2-1a and huEPOR-3-2 were isolated. Phage DNA may be prepared from each, by methods described in Maniatis, et al., Molecular Cloning: A Laboratory Manual (1982). Phage DNA is then treated with restriction enzyme Sau3A1, phosphated with calf alkaline phosphatase, phenol extracted and coprecipitated with 20 mg of BamHI cut M13mp8 DNA. The precipitated DNA is pelleted by centrifugation and redissolved in 50 ul of ligase buffer containing T4 DNA ligase and

incubated for 3 hours at 16°C. 5 ul of this reaction mixture is used to transform E. coli strain JM101/T61. The plaques are screened using the Benton and Davis procedure and probed with the 1.8 kb murine erythropoietin receptor cDNA fragment. Phage plaques exhibiting hybridization are isolated and single stranded phage DNA is prepared for use a DNA sequencing template. The sequence of each recombinant M13 phage's genomic human DNA fragment is determined by the dideoxy chain termination technique described by Sanger, et al., PNAS 74:5463-5467 (1977). Commercially available primers were used which flanked the human genomic DNA insertion site in M13. A composite human genomic DNA nucleotide sequence can be constructed from the nucleic acid sequence of various phage plaques exhibiting hybridization (at 48°C and standard hybridization mixture). The complete human genomic gene for the erythropoietin receptor should contain nucleic acid sequence with ATG at the 5' end and a stop codon at the 3' end such that the nucleic acid sequences are analogous and similar to the nucleic acid sequence in between nucleotides 28 and 1551 of the mouse erythropoietin receptor sequence depicted in Figure 1. The human genomic DNA sequence may show breaks between region of close similarity to the murine erythropoietin sequence. These breaks are characteristic of the intron/exon structure of mammalian genomic DNA.

To determine if the human genomic DNA sequence encodes a functional erythropoietin receptor, it is necessary to link the gene to the appropriate expression elements and introduce the construct into mammalian cell. Suitable expression vectors include pXM, pMT2, pMT21, etc.

From the M13 phages containing the genomic human gene for the erythropoietin receptor, a single fragment of DNA containing the entire human erythropoietin receptor coding region can be assembled and contained within the plasmid pXM such that the human erythropoietin receptor gene is in operative association with the mammalian expression elements of pXM. The pXM-human erythropoietin-R plasmid can be transfected into COS cells by conventional means to yield an active erythropoietin binding protein. The pXM human erythropoietin plasmid can be used to generate recombinant cell line constitutively expressing a human erythropoietin receptor or through the use of regulatable gene expression elements construct a recombinant cell line expressing the human erythropoietin receptor in a regulatable fashion.

Isolation of the Human cDNA Gene for an Erythropoietin Receptor

The human cDNA gene for the erythropoietin receptor can be isolated from a cDNA library of mRNA from the recombinant call line described above or from a human tissue or cell line. Examples of human tissues which may express the human erythropoietin receptor are fetal spleen, fetal liver, bone marrow, erythroleukemia cells, and established erythroleukemia cell lines such as OCIM1 (Broudy et al., PNAS, 85:6513-6517 (1988); HEL (Martin and Papayannopoulou, Science, 216:1233-1235 (1982); KMOE (Kaku, et al., Blood, 64:314-317 (1980); K562 (Andersson et al., Nature, 278:364-365 (1979) and JK-1 (Hitomi et al., BBRC, 154:902-909 (1988). By Northern analysis using the 1.8 kb mouse EPO-R cDNA fragment or the partial human

genomic clone as a probe, in standard hybridization buffer at a hybridization temperature of 48°C, the tissue and cell lines can be tested for the presence of a mRNA encoding a human EPO-R.

mRNA is prepared from each tissue and cell line source by standard techniques and enriched for mRNA by chromatography on oligo (dT) cellulose. The isolated mRNA is subjected to electrophoresis through an agarose gel containing formaldehyde (Maniatis et al., Molecular Cloning: A Laboratory Manual (1982)) and the mRNA is transferred to nitrocellulose. The nitrocellu-lose filter is hybridized with the murine receptor cDNA fragment. The erythropoietin receptor cDNA fragment is radiolabeled to a specific activity of 10°C for 18 hours. The filter is washed at 55°C, 0.5XSSC for 1 hour, and then autoradiographed. A discrete hybridization signal is an indication that the tissue or cell line is a source of the mRNA for a human erythropoietin receptor.

The appropriate mRNA can be used to construct a cDNA library in either the plasmid pXM or in a phage vector such as Lambda Zap (Stratagene). The mRNA is converted to double stranded cDNA (Gubler and Hoffman, Gene, 25:263-269 (1982) or by methods described in Maniatis, et al., Molecular Cloning: A Laboratory Manual (1982) and adapted with synthetic oligonucleotides as described in Yang, et al., Cell, 47:3 (1986), or as in Maniatis, et al., and inserted into the plasmid vector pXM or phage vector Lambda Zap.

The plasmid or phage cDNA library can be screened at 48°C using the erythropoietin receptor cDNA fragment as a probe as described in Toole et al. (U.S. Patent No. 4,757,006).

For example, using the murine EPO-R clone 190 as a hybridization probe, we identified several hybridizing clones in a cDNA library prepared from a human erythroleukemia cell line. Two of those clones were sequenced and used to construct (based at least in part on the murine sequence) a composite full-length intronless DNA sequence encoding human EPO-R protein in plasmid pMT21. The composite DNA encodes an EPO-R protein which binds to EPO and should transduce signal into cells expressing it. The human DNA and deduced peptide sequences are shown in Figure 2. As can be readily determined, the human and murine sequences are strongly homologous, sharing greater than about 85% and about 80% homology at the DNA and peptide levels, respectively. We also note that screening a human fetal liver library with the murine clone also led to the identification of several hybridization positives.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

1. An isolated DNA sequence encoding all or a portion of a cell surface receptor for erythropoietin, or a functional equivalent thereof.

2. An isolated DNA sequence consisting essentially of the DNA sequence encoding all or a portion of a cell surface receptor for erythropoietin, or a functional equivalent thereof.

3. An cDNA or other intronless DNA sequence encoding all or a portion of a cell surface receptor for erythropoietin.

4. A DNA sequence of Claims 1, 2 or 3 which is mammalian.

5. A DNA sequence of Claim 4 which is human or murine.

6. A DNA sequence of any of Claims 1-5 which is lacking part or all of the transmembrane region.

7. An isolated or intronless DNA sequence encoding part or all of the peptide sequence of Figures 1 or 2.

8. A recombinant DNA vector containing a DNA sequence of any of Claims 1-7.

9. A host cell containing and capable of expressing a DNA sequence according to any of Claims 1-7.

10. Isolated erythropoietin receptor protein free from other proteins with which it is associated in nature.

11. An isolated polypeptide encoded by a DNA sequence encoding all or a portion of a cell surface receptor for erythropoietin, or the functional equivalent thereof.

12. An isolated polypeptide encoded by all or a portion of a DNA sequence of any of Claims 1-7.

13. An antibody or fragment thereof capable of specific binding towards an erythropoietin receptor of this invention.

14. An antibody or fragment thereof according to claim 13, the presence of which is capable of supporting the growth of an erythropoietin-dependent cell line.

15. A pharmaceutical composition for the treatment of anemia comprising a therapeutically effective amount of an antibody or fragment thereof of Claim 14 in admixture with a pharmaceutically acceptable carrier.

16. A method for purifying erythropoietin which comprises contacting an erythropoietin-containing sample with an erythropoietin receptor protein under conditions permitting the erythropoietin to bind to the erythropoietin receptor protein to form a complex, separating the complex from the remainder of the sample, and recovering the bound erythropoietin from the separated complex.

17. A method of purifying erythropoietin according to Claim 16, wherein the erythropoietin is immobilized.

18. An isolated human RNA transcript encoding all or a portion of a cell surface receptor for erythropoietin or the functional equivalent thereof.

19. A method for identifying a therapeutic agent for the treatment of anemia comprising:
(a) contacting potential such agents with an erythropoietin receptor protein capable of binding to erythropoietin, the contacting being under conditions which would permit binding of erythropoietin to the receptor protein;
(b) identifying those agents which detectably bind to the receptor protein;
(c) screening agents so identified for <u>in vitro</u> or <u>in vivo</u> stimulation of erythropoiesis; and,
(d) identifying the agent or agents which exhibit erythropoiesis.

FIGURE 1

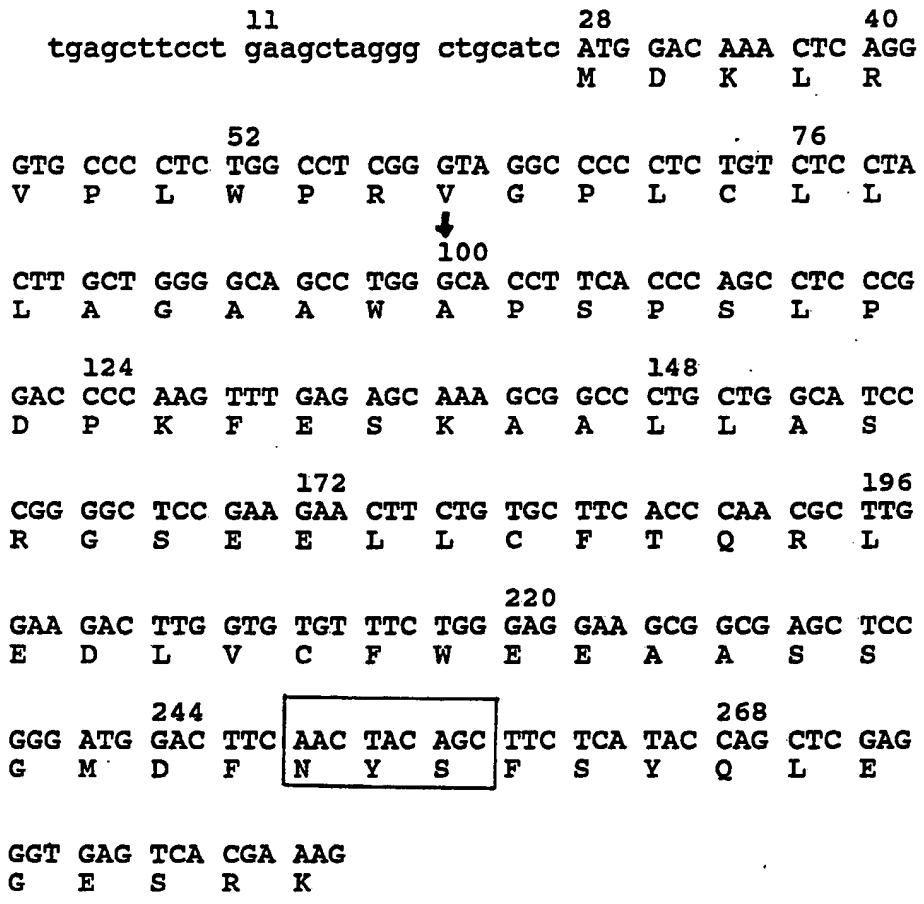
11
28
40
tgagcttcct gaagctaggg ctgcatc ATG GAC AAA CTC AGG
M D K L R
52
76
GTG CCC CTC TGG CCT CGG GTA GGC CCC CTC TGT CTC CTA
V P L W P R V G P L C L L
100
CTT GCT GGG GCA GCC TGG GCA CCT TCA CCC AGC CTC CCG
L A G A A W A P S P S L P
124
148
GAC CCC AAG TTT GAG AGC AAA GCG GCC CTG CTG GCA TCC
D P K F E S K A A L L A S
172
196
CGG GGC TCC GAA GAA CTT CTG TGC TTC ACC CAA CGC TTG
R G S E E L L C F T Q R L
220
GAA GAC TTG GTG TGT TTC TGG GAG GAA GCG GCG AGC TCC
E D L V C F W E E A A S S
244
268
GGG ATG GAC TTC AAC TAC AGC TTC TCA TAC CAG CTC GAG
G M D F N Y S F S Y Q L E
GGT GAG TCA CGA AAG
G E S R K

FIGURE 1A

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292                                                316
TCA TGT AGC CTG CAC CAG GCT CCC ACC GTC CGC GGC TCC
S   C   S   L   H   Q   A   P   T   V   R   G   S

                340                                368
GTG CGT TTC TGG TGT TCA CTG CCA ACA GCG GAC ACA TCG
V   R   F   W   C   S   L   P   T   A   D   T   S

                                388
AGT TTT GTG CCG CTG GAG CTG CAG GTG ACG GAG GCG TCC
S   F   V   P   L   E   L   Q   V   T   E   A   S

    412                                    436
GGT TCT CCT CGC TAT CAC CGC ATC ATC CAT ATC AAT GAA
G   S   P   R   Y   H   R   I   I   H   I   N   E

                460                                484
GTA GTG CTC CTG GAC GCC CCC GCG GGG CTG CTG GCG CGC
V   V   L   L   D   A   P   A   G   L   L   A   R

                                    508
CGG GCA GAA GAG GGC AGC CAC GTG GTG CTG CGC TGG CTG
R   A   E   E   G   S   H   V   V   L   R   W   L

        532                                556
CCA CCT CCT GGA GCA CCT ATG ACC ACC CAC ATC CGA TAT
P   P   P   G   A   P   M   T   T   H   I   R   Y

                    580
GAA GTG GAC GTG TCG GCA GGC AAC CGG GCA GGA GGG ACA
E   V   D   V   S   A   G   N   R   A   G   G   T

604                                        628
CAA AGG GTG GAG GTC CTG GAA GGC CGC ACT GAG TGT GTT
Q   R   V   E   V   L   E   G   R   T   E   C   V

                652
CTG AGC AAC CTG CGG GGC GGG ACG CGC TAC ACC
L   S   N   L   R   G   G   T   R   Y   T
```

FIGURE 1B

```
676                                                 700
TTC GCT GTT CGA GCG CGC ATG GCC GAG CCG AGC TTC AGC
F   A   V   R   A   R   M   A   E   P   S   F   S

                724                                 748
GGA TTC TGG AGT GCC TGG TCT GAG CCC GCG TCA CTA CTG
G   F   W   S   A   W   S   E   P   A   S   L   L

                            772
ACC GCT AGC GAC CTG GAC CCT CTC ATC TTG ACG CTG TCT
T   A   S   D   L   D   P   L   I   L   T   L   S

    796                                 820
CTC ATT CTG GTC CTC ATC TCG CTG TTG CTG ACG GTT CTG
L   I   L   V   L   I   S   L   L   L   T   V   L

                    844                             868
GCC CTG CTG TCC CAC CGC CGG ACT CTG CAG CAG AAG ATC
A   L   L   S   H   R   R   T   L   Q   Q   K   I

                                892
TGG CCT GGC ATC CCA AGC CCA GAG AGC GAG TTT GAG GGT
W   P   G   I   P   S   P   E   S   E   F   E   G

        916                                 940
CTC TTC ACC ACC CAC AAG GGT AAC TTC CAG CTG TGG CTG
L   F   T   T   H   K   G   N   F   Q   L   W   L

                        964
CTG CAG CGT GAT GGT TGT CTG TGG TGG AGC CCG GGC AGC
L   Q   R   D   G   C   L   W   W   S   P   G   S

988                                 1012
TCC TTC CCT GAG GAT CCA CCT GCC CAC CTA GAG GTC CTC
S   F   P   E   D   P   P   A   H   L   E   V   L

                1036
TCA GAG CCA CGC TGG GCA GTG ACT CAG GCT GGG
S   E   P   R   W   A   V   T   Q   A   G
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FIGURE 1C

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1060                                                1084
GAC CCA GGG GCA GAT GAT GAG GGG CCC TTA CTG GAG CCG
D   P   G   A   D   D   E   G   P   L   L   E   P

            1108                                        1132
GTG GGC AGT GAG CAT GCC CAG GAC ACC TAC TTG GTA TTG
V   G   S   E   H   A   Q   D   T   Y   L   V   L

                                1156
GAT AAG TGG TTG CTG CCC CGG ACC CCA TGC AGT GAG
D   K   W   L   L   P   R   T   P   C   S   E

        1180                                        1204
AAC CTC TCA GGG CCT GGG GGC AGT GTG GAC CCT GTG ACT
N   L   S   G   P   G   G   S   V   D   P   V   T

                        1228
ATG GAT GAA GCT TCA GAA ACA TCT TCC TGC CCG TCT GAC
M   D   E   A   S   E   T   S   S   C   P   S   D

1252                                                1276
TTG GCC TCA AAG CCC AGG CCA GAG GGC ACC TCA CCT TCC
L   A   S   K   P   R   P   E   G   T   S   P   S

            1300                                        1324
AGC TTT GAG TAC ACC ATC CTG GAC CCC AGC TCT CAG CTC
S   F   E   Y   T   I   L   D   P   S   S   Q   L

    *                       1348
CTG TGC CCT CGG GCA CTG CCT CCC GAG CTA CCT CCC ACT
L   C   P   R   A   L   P   P   E   L   P   P   T

    1372                                        1396
CCA CCT CAC TTG AAG TAC CTA TAC CTT GTG GTG TCC GAT
P   P   H   L   K   Y   L   Y   L   V   V   S   D

                    1420
TCT GGC ATC TCA ACA GAT TAC AGT TCG GGG GGC TCT
S   G   I   S   T   D   Y   S   S   G   G   S
```

FIGURE 1D

```
1444                                                    1468
CAG GGA GTC CAC GGG GAC TCA TCT GAT GGC CCC TAC TCC
Q   G   V   H   G   D   S   S   D   G   P   Y   S

                1492                                            1516
CAC CCC TAT GAG AAC AGC CTT GTC CCA GAC TCA GAG CCT
H   P   Y   E   N   S   L   V   P   D   S   E   P

                                    1540
CTG CAT CCC GGC TAT GTG GCC TGC TCC TAG gactccagcc
L   H   P   G   Y   V   A   C   S

1562                        1582
tacaacgtct tgaacgggat tggtgaagcc ata cttaaag

1602                        1622
tcagagctga ccttggccct ctgagcagga agagacagcc

1642                        1662
ttgcaatgtt aagattaaga gttatctgtc tgtatataga

1682                        1702
aatatatata tatatcgatt tttctacc tt gaaaaaaaaa

1722
aaaaaaaaaa aaaaaaaaaa
```

FIGURE 2

```
         10          20          30          40          50          60          70
CTCGAGCTGC AGAGCTAGCT CTGCAGCTCG CTGCAGAGCT CAGCTGCGTC CGGCGGAGGC AGCTGCTGAC

         80          90          100         110         120         130         140
CCAGCTGTGG ACTGTGCCGG GGGCGGGGGA CGGAGGGGCA GGAGCCCTGG GCTCCCCGTG GCGGGGGCTG

              150         159         168         177         186
              >
TATC ATG GAC CAC CTC GGG GCG TCC CTC TGG CCC CAG GTC GGC TCC CTT TGT
     M   D   H   L   G   A   S   L   W   P   Q   V   G   S   L   C

195         204         213         222         231         240
CTC CTG CTC GCT GGG GCC GCC TGG GCG CCC CCG CCT AAC CTC CCG GAC CCC AAG
L   L   L   A   G   A   A   W   A   P   P   P   N   L   P   D   P   K

249         258         267         276         285         294
TTC GAG AGC AAA GCG GCC TTG CTG GCG GCC CGG GGG CCC GAA GAG CTT CTG TGC
F   E   S   K   A   A   L   L   A   A   R   G   P   E   E   L   L   C
```

FIGURE 2A

303			312			321			330			339			348		
TTC	ACC	GAG	CGG	TTG	GAG	GAC	TTG	GTG	TGT	TTC	TGG	GAG	GAA	GCG	GCG	AGC	GCT
F	T	E	R	L	E	D	L	V	C	F	W	E	E	A	A	S	A
357			366			375			384			393			402		
GGG	GTG	GGC	CCG	GGC	AAC	TAC	AGC	TTC	TCC	TAC	CAG	CTC	GAG	GAT	GAG	CCA	TGG
G	V	G	P	G	N	Y	S	F	S	Y	Q	L	E	D	E	P	W
411			420			429			438			447			456		
AAG	CTG	TGT	CGC	CTG	CAC	CAG	GCT	CCC	ACG	GCT	CGT	GGT	GCG	GTG	CGC	TTC	TGG
K	L	C	R	L	H	Q	A	P	T	A	R	G	A	V	R	F	W
465			474			483			492			501			510		
TGT	TCG	CTG	CCT	ACA	GCC	GAC	ACG	TCG	AGC	TTC	GTG	CCC	CTA	GAG	TTG	CGC	GTC
C	S	L	P	T	A	D	T	S	S	F	V	P	L	E	L	R	V
519			528			537			546			555			564		
ACA	GCA	GCC	TCC	GGC	GCT	CCG	CGA	TAT	CAC	CGT	GTC	ATC	CAC	ATC	AAT	GAA	GTA
T	A	A	S	G	A	P	R	Y	H	R	V	I	H	I	N	E	V
573			582			591			600			609			618		
GTG	CTC	CTA	GAC	GCC	CCC	GTG	GGG	CTG	GTG	GCG	CGG	TTG	GCT	GAC	GAG	AGC	GGC
V	L	L	D	A	P	V	G	L	V	A	R	L	A	D	E	S	G
627			636			645			654			663			672		
CAC	GTA	GTG	TTG	CGC	TGG	CTC	CCG	CCG	CCT	GAG	ACA	CCC	ATG	ACG	TCT	CAC	ATC
H	V	V	L	R	W	L	P	P	P	E	T	P	M	T	S	H	I

FIGURE 2B

681			690			699			708			717			726		
CGC	TAC	GAG	GTG	GAC	GTC	TCG	GCC	GGC	AAC	GGC	GCA	GGG	AGC	GTA	CAG	AGG	GTG
R	Y	E	V	D	V	S	A	G	N	G	A	G	S	V	Q	R	V
735			744			753			762			771			780		
GAG	ATC	CTG	GAG	GGC	CGC	ACC	GAG	TGT	GTG	CTG	AGC	AAC	CTG	CGG	GGC	CGG	ACG
E	I	L	E	G	R	T	E	C	V	L	S	N	L	R	G	R	T
789			798			807			816			825			834		
CGC	TAC	ACC	TTC	GCC	GTC	CGC	GCG	CGT	ATG	GCT	GAG	CCG	AGC	TTC	GGC	GGC	TTC
R	Y	T	F	A	V	R	A	R	M	A	E	P	S	F	G	G	F
843			852			861			870			879			888		
TGG	AGC	GCC	TGG	TCG	GAG	CCT	GTG	TCG	CTG	CTG	ACG	CCT	AGC	GAC	CTG	GAC	CCC
W	S	A	W	S	E	P	V	S	L	L	T	P	S	D	L	D	P
897			906			915			924			933			942		
CTC	ATC	CTG	ACG	CTC	TCC	CTC	ATC	CTC	GTG	GTC	ATC	CTG	GTG	CTG	CTG	ACC	GTG
L	I	L	T	L	S	L	I	L	V	V	I	L	V	L	L	T	V
951			960			969			978			987			996		
CTC	GCG	CTG	CTC	TCC	CAC	CGC	CGG	GCT	CTG	AAG	CAG	AAG	ATC	TGG	CCT	GGC	ATC
L	A	L	L	S	H	R	R	A	L	K	Q	K	I	W	P	G	I

FIGURE 2C

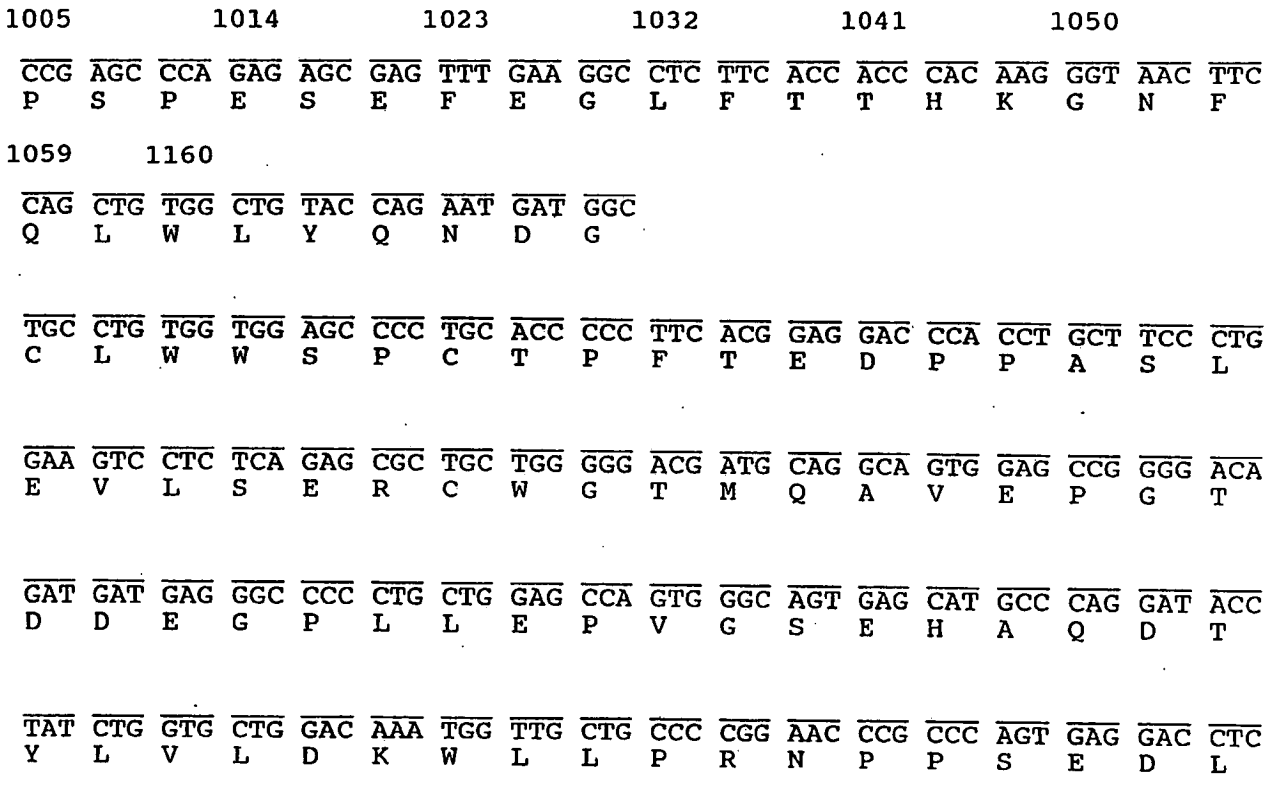
1005 1014 1023 1032 1041 1050
CCG AGC CCA GAG AGC GAG TTT GAA GGC CTC TTC ACC ACC CAC AAG GGT AAC TTC
P S P E S E F E G L F T T H K G N F
1059 1160
CAG CTG TGG CTG TAC CAG AAT GAT GGC
Q L W L Y Q N D G
TGC CTG TGG TGG AGC CCC TGC ACC CCC TTC ACG GAG GAC CCA CCT GCT TCC CTG
C L W W S P C T P F T E D P P A S L
GAA GTC CTC TCA GAG CGC TGC TGG GGG ACG ATG CAG GCA GTG GAG CCG GGG ACA
E V L S E R C W G T M Q A V E P G T
GAT GAT GAG GGC CCC CTG CTG GAG CCA GTG GGC AGT GAG CAT GCC CAG GAT ACC
D D E G P L L E P V G S E H A Q D T
TAT CTG GTG CTG GAC AAA TGG TTG CTG CCC CGG AAC CCG CCC AGT GAG GAC CTC
Y L V L D K W L L P R N P P S E D L

FIGURE 2D

CCA	GGG	CCT	GGT	GGC	AGT	GTG	GAC	ATA	GTG	GCC	ATG	GAT	GAA	GGC	TCA	GAA	GCA
P	G	P	G	G	S	V	D	I	V	A	M	D	E	G	S	E	A
TCC	TCC	TGC	TCA	TCT	GCT	TTG	GCC	TCG	AAG	CCC	AGC	CCA	GAG	GGA	GCC	TCT	GCT
S	S	C	S	S	A	L	A	S	K	P	S	P	E	G	A	S	A
GCC	AGC	TTT	GAG	TAC	ACT	ATC	CTG	GAC	CCC	AGC	TCC	CAG	CTC	TTG	CGT	CCA	TGG
A	S	F	E	Y	T	I	L	D	P	S	S	Q	L	L	R	P	W
ACA	CTG	TGC	CCT	GAG	CTG	CCC	CCT	ACC	CCA	CCC	CAC	CTA	AAG	TAC	CTG	TAC	CTT
T	L	C	P	E	L	P	P	T	P	P	H	L	K	Y	L	Y	L
GTG	GTA	TCT	GAC	TCT	GGC	ATC	TCA	ACT	GAC	TAC	AGC	TCA	GGG	GAC	TCC	CAG	GGA
V	V	S	D	S	G	I	S	T	D	Y	S	S	G	D	S	Q	G
GCC	CAA	GGG	GGC	TTA	TCC	GAT	GGC	CCC	TAC	TCC	AAC	CCT	TAT	GAG	AAC	AGC	CTT
A	Q	G	G	L	S	D	G	P	Y	S	N	P	Y	E	N	S	L

FIGURE 2E

```
ATC CCA GCC GCT GAG CCT CTG CCC CCC AGC TAT GTG GCT TGC TCT> TAGGACACCA
I   P   A   A   E   P   L   P   P   S   Y   V   A   C   S

GGCTGCAGAT GATCAGGGAT CCAATATGAC TCAGAGAACC AGTGCAGACT CAAGACTTAT GGAACAGGGA

TGGCGAGGCC TCTCTCAGGA GCAGGGGCAT TGCTGATTTT GTCTGCCCAA TCCATCCTGC TCAGGAAACC

ACAACCTTGC AGTATTTTTA AATATGTATA GTTTTTTTGC TGCAGAGCTA GCTCTGCAGC TCGAG
```

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/00635

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/12, C 12 N 5 /10, C 12 P 21/02, A 61 K 39/395, A 61 K 37/02, A 61 K 37/24, G 01 N 33/74, C 12 Q 1/18, ./.

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	Cell, vol. 57, 21 April 1989, Cell Press, (Cambridge, Mass., US), A.D. D'Andrea et al.: "Expression cloning of the murine erythropoietin receptor", pages 277-285, see the whole article (cited in the application)	1-12,18
A	The Journal of Biological Chemistry, vol. 262, no. 29, 15 October 1987, The American Society for Biochemistry and Molecular Biology, Inc., (Baltimore, US), P. Mayeux et al.: "The erythropoietin receptor of rat erythroid progenitor cells", pages 13985-13990, see abstract (cited in the application)	10

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th May 1990	26. 06. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/00635

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IPC[5]: C 12 P 21/08, C 07 K 15/00

II. FIELDS SEARCHED

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A	Biochemical and Biophysical Research Communications, vol. 154, no. 3, 15 August 1988, Academic Press, Inc., K. Hitomi et al.: "Erythropoietin receptor of a human leukemic cell line with erythroid characteristics", pages 902-909, see pages 906-909: "Discussion"	10

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report

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EUROPEAN PATENT OFFICE	